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Dominance of *Vibrio fischeri* in Secreted Mucus outside the Light Organ of *Euprymna scolopes*: the First Site of Symbiont Specificity

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Previous studies of the *Euprymna scolopes-Vibrio fischeri* symbiosis have demonstrated that, during colonization, the hatchling host secretes mucus in which gram-negative environmental bacteria amass in dense aggregations outside the sites of infection. In this study, experiments with green fluorescent protein-labeled symbiotic and nonsymbiotic species of gram-negative bacteria were used to characterize the behavior of cells in the aggregates. When hatchling animals were exposed to 10^3 to 10^6 *V. fischeri* cells/ml added to natural seawater, which contains a mix of approximately 10^6 nonspecific bacterial cells/ml, *V. fischeri* cells were the principal bacterial cells present in the aggregations. Furthermore, when animals were exposed to equal cell numbers of *V. fischeri* (either a motile or a nonmotile strain) and either *Vibrio parahaemolyticus* or *Photobacterium leiognathi*, phylogenetically related gram-negative bacteria that also occur in the host's habitat, the symbiont cells were dominant in the aggregations. The presence of *V. fischeri* did not compromise the viability of these other species in the aggregations, and no significant growth of *V. fischeri* cells was detected. These findings suggested that dominance results from the ability of *V. fischeri* either to accumulate or to be retained more effectively within the mucus. Viability of the *V. fischeri* cells was required for both the formation of tight aggregates and their dominance in the mucus. Neither of the *V. fischeri* quorum-sensing compounds accumulated in the aggregations, which suggested that the effects of these small signal molecules are not critical to *V. fischeri* dominance. Taken together, these data provide evidence that the specificity of the squid-vibrio symbiosis begins early in the interaction, in the mucus where the symbionts aggregate outside of the light organ.

In many symbioses between bacteria and animals or plants, the bacterial partner forms a monospecific association with the host that is reestablished with each new generation (12). Such associations form with fidelity, although a given as-yet-uncolonized juvenile host may be exposed to a vast array of other potential microbial partners that are present in the environment. The mechanisms by which this specificity is achieved, as well as the relative contributions of the host and the symbiont to these processes, remain poorly understood for most animal associations. In contrast, studies of the leguminous plant associations with nitrogen-fixing rhizobia, in which dozens of genes orchestrate the complex molecular interplay essential for colonization, have provided a dramatic example of how complex the process of ensuring specificity can be (24).

The Hawaiian squid *Euprymna scolopes* forms a persistent association with the gram-negative luminous bacterium *Vibrio fischeri* (11, 27). Studies of this experimental model of animal-bacterial associations have provided evidence to date that, similar to the case for the legume-rhizobium symbioses, specificity is mediated by a series of biomechanical, biochemical, and molecular determinants of the host and its bacterial partner (4, 13, 14, 21, 26, 29). In the squid-vibrio system, host embryogenesis is completed in the absence of the bacterial partner (15, 18). However, during the embryonic period specificity determinants develop that promote the establishment of the two-partner symbiosis within hours of the host's hatching (17, 18). During this relatively brief posthatch period, the host and symbiont, which interact within the context of $\sim 10^6$ nonsymbiotic bacteria per ml that co-occur in the surrounding seawater (1), must encounter and recognize one another. Further, the interactions of the partners must proceed in such a way as to exclude other potential partners; in the absence of *V. fischeri*, no other bacteria naturally colonize host tissues (15). In the final steps of the colonization process, the symbionts migrate to one of six pores on the light organ surface, enter the pores, and travel up ducts that lead to six independent epithelium-lined crypts (17). These crypts are the sites of the persistent colonization of host tissues by the bacterial symbiont.

Studies of the process of colonization have revealed the mechanism by which symbionts are harvested by the host (20, 21). Cells of a ciliated field on the surface of the nascent light organ secrete mucus. The induction of this secretory activity is relatively nonspecific; exposure to natural seawater, a variety of both gram-negative and gram-positive bacteria, or bacterial peptidoglycan results in copious mucus secretion. Association with host-secreted mucus is more specific; i.e., only gram-negative bacteria, either symbiotic or nonsymbiotic strains, are able to aggregate in the mucus. Bacterium-laden mucus is suspended above the sites of colonization by the activity of the cilia of the superficial epithelium, and when the aggregated cells are *V. fischeri*, they eventually migrate into host tissues.

These initial studies characterized the behavior of pure cultures of bacteria in the induction of mucus shedding and the aggregation of bacteria within these secretions. Because *V. fischeri* cells represent only about 0.1% of the bacterial cells in

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the surrounding seawater (8) and only *V. fischeri* is capable of the eventual colonization of the light organ crypts (15), the symbiont cells must have mechanisms to establish dominance at some point in the colonization process. In the present study, we sought to determine where along this process the specific interaction between the host squid and *V. fischeri* begins. Our data provide evidence that the specificity of this association begins during the aggregation process, prior to entry into the light organ crypt spaces.

MATERIALS AND METHODS

General procedures. The host animals were collected and maintained as previously described (3). For assessment of bacterial aggregation in host-shed mucus, a given seawater sample was prepared and dispensed in 1-ml aliquots into the wells of 24-well microtiter dishes, and an individual hatchling squid was added to each well. After a 3- to 4-h incubation, a time period previously shown to result in maximal aggregation (21), animals were anesthetized in 2% ethanol in filter-sterilized seawater (FSSW) and ventrally dissected. Bacterial aggregations in association with the light organ were observed by laser scanning confocal microscopy (LSM) and differential interference contrast (DIC) microscopy on a Zeiss 510 laser scanning confocal microscope.

Cultured bacterial cells were grown in seawater tryptone (SWT) medium (2) to mid-log phase, cell density was determined spectrophotometrically, and this value was used to calculate the inoculum size for a given experiment. The inoculum size was confirmed by plating the cells on SWT agar.

Competition of *V. fischeri* with other bacteria for space within the aggregations. To determine whether *V. fischeri* is able to outcompete other bacteria for space within the aggregations under relatively natural conditions, green fluorescent protein (GFP)-labeled cells of *V. fischeri* were culture grown and diluted to final concentrations ranging from 10^3 to 10^6 CFU/ml in natural, unfiltered seawater, which contains a background of approximately 10^6 CFU of a variety of nonsymbiotic gram-negative and gram-positive bacteria per ml (1). The seawater used for these experiments does not naturally contain sufficient quantities of *V. fischeri* to cause colonization of the light organ; i.e., it contains fewer than approximately 50 cells/ml of seawater (J. McCann and E. Ruby, personal communication).

Experiments were performed to (i) determine whether any observed dominance of *V. fischeri* was due to the pregrowth of the cells under culture conditions and (ii) define the extent of the bacterial specificity within the aggregations. For these experiments unlabeled *V. fischeri* ES114 (4) and either GFP-labeled *Vibrio parahaemolyticus* (KNH1) (21) or *Photobacterium leiognathi* (KNH6) (E. Stabb, unpublished data) were grown and added to FSSW, each at 10^6 CFU/ml. Cross-inoculation studies under culture conditions produced no evidence that any of the strains inhibited the growth of the other strains. A reciprocal infection with unlabeled *V. parahaemolyticus* and GFP-labeled *V. fischeri* was also performed to determine whether GFP labeling affected the outcome of these competition experiments. In addition, control incubations with each bacterial species (labeled or unlabeled) alone were performed. To determine whether motility in *V. fischeri* may confer a competitive advantage for aggregation, coincubations were also performed with a *V. fischeri* strain defective in motility (NM200, a flagellated but nonmotile strain [4]) with cells of motile *V. parahaemolyticus.*

Determinations of symbiont growth rate within the aggregations. To determine whether the dominance of *V. fischeri* in the aggregations is due to cell growth, hatchling squid were infected with cells of *V. fischeri* ES114 in the presence of nalidixic acid. Nalidixic acid is an inhibitor of bacterial DNA synthesis that allows cell growth but inhibits cell division; growing cells exposed to this antibiotic do not divide and thus form elongated filaments (7). In preliminary experiments with culture-grown *V. fischeri* cells, we determined that 25 μg of nalidixic acid per ml was the optimal concentration for the visualization of filaments. Bacteria were grown in culture to log phase and then exposed to the animals at 10^3 cells/ml of FSSW containing nalidixic acid at a concentration of 25 μg/ml for 3 to 4 h. Using LSM, *V. fischeri* cell growth within the aggregations was assessed by optical sectioning of the aggregations, and lengths of treated and untreated bacterial cells were compared.

Role of viability of cells in the aggregates. Experiments were performed to assess whether any observed dominance in the mucus aggregations requires viability of *V. fischeri* cells and whether *V. fischeri* compromises the viability of other bacterial cells in the aggregates. First, we determined whether symbiotic and nonsymbiotic species are equally viable within the mucus matrix of the aggregations. In these experiments, we evaluated the proportion of viable cells in aggregations of animals that had been exposed to either culture-grown *V. fischeri* ES114, *V. parahaemolyticus* KNH1, or *P. leiognathi* KNH6 cells. After a 3- to 4-h incubation with the cells and 30 min prior to dissection, the animals were placed in FSSW containing 1 μM propidium iodide. This fluorochrome stains the nucleic acids of dead and dying bacterial cells that have compromised membranes (5). Squid were viewed by LSM, and the ratio of living to dead bacterial cells within the aggregations was determined.

We then determined whether the presence of *V. fischeri* compromises the viability of the *V. parahaemolyticus* or *P. leiognathi* cells within the aggregations. In these trials, after a 2-h incubation with a 1:1 ratio of the unlabeled *V. fischeri* to the GFP-labeled nonsymbiotic species, the animals were treated as described above to determine the ratio of live to dead symbiotic and nonsymbiotic cells.

We also performed a series of experiments to assess whether *V. fischeri* viability is essential for its dominance in the aggregations. First, hatchling squid were incubated with either heat-inactivated or azide-treated *V. fischeri* ES114 cells to determine whether normal, tight aggregations would form under these conditions. Animals were then coincubated with *V. parahaemolyticus* and either viable or compromised (heat-inactivated or azide-treated) *V. fischeri* cells. To heat inactivate *V. fischeri* cells, cultures were grown to mid-log phase in SWT medium and then incubated at 45°C for 30 min. Sodium azide-treated *V. fischeri* cells were also grown to mid-log phase in SWT medium but instead were exposed to 0.1% sodium azide for 1 h, followed by rinsing 10 times in FSSW before inoculation. To determine the efficiency of heat or azide inactivation, in each case, a subset of the treated cells was viewed by fluorescence microscopy to ensure that motility had ceased and that the cellular GFP was still fluorescent. In addition, aliquots of the cells were plated on SWT agar and CFU were determined. Propidium iodide, used as described above, was also used to assess viability.

Assay for accumulation of the signal molecules VAI-1 and VAI-2 within the aggregations. For studies of the behavior of autoinducer molecules in the mucus aggregates, animals were exposed to cells of the nonmotile *V. fischeri* strain NM200 (a nonflagellated strain) at 10^6 cells/ml in FSSW. This mutant strain does not migrate into the ducts or colonize the crypts (4, 21). However, when the host is exposed to 10^6 cells of *V. fischeri* NM200 per ml, the cells form aggregations with densities similar to the symbiont concentration normally encountered in a fully colonized and luminous light organ, i.e., densities that result in the autoinduction of luminescence in the crypts. At 3 or 4 h after exposure to bacteria, the animals were rinsed in FSSW and exposed either to FSSW alone or to FSSW containing 100 ng of the *V. fischeri* autoinducer *N*-3-oxohexanoyl-L-homoserine lactone (VAI-1) (Sigma-Aldrich, St. Louis, Mo.) or octanoyl-L-homoserine lactone (VAI-2) (Aurora Biosciences, San Diego, Calif.) per ml. Following a 30-min incubation with VAI-1 or VAI-2, the presence or absence of luminescence of aggregated bacteria was measured with a TD-20/20 luminometer (Turner Instruments, Sunnyvale, Calif.). The animals were then dissected, and the presence of robust bacterial aggregations was confirmed by LSM.

RESULTS

Specificity within the aggregations. In these experiments, when hatchling squid were exposed to unfiltered seawater devoid of *V. fischeri*, mucus aggregates containing nonsymbiotic bacteria were observed, similar to those reported previously (20, 21). However, when hatchling squid were exposed for 4 h to unfiltered environmental seawater to which *V. fischeri* cells had been added at concentrations ranging from 10^3 to 10^6 cells/ml, *V. fischeri* cells formed tightly packed aggregations (Fig. 1A), irrespective of their proportion relative to nonsymbiotic bacteria in the seawater. Under these conditions, *V. fischeri* cells accounted for >99% of the cells in the aggregations. These data suggested that *V. fischeri* was somehow competitively dominant for space within the aggregations.

To eliminate the possibility that cultured cells had an advantage over the cells naturally present in seawater, we incubated host animals for 4 h with a 1:1 mix of *V. fischeri* and either *V. parahaemolyticus* or *P. leiognathi*. These nonsymbiotic species formed tight aggregations in the absence of *V. fischeri*. In the competition experiments, *V. fischeri* cells were able to outcompete the nonsymbiotic species for space within the ag-

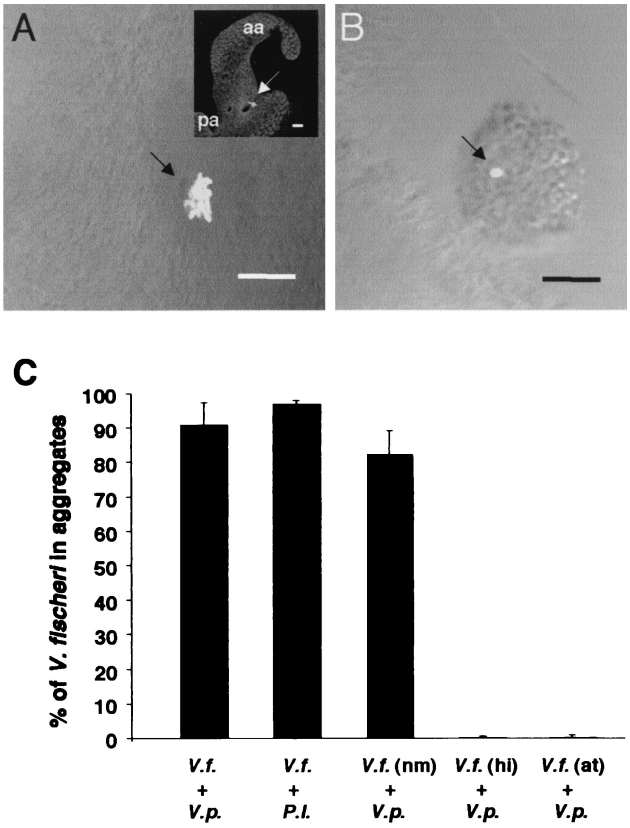

FIG. 1. Specificity of *V. fischeri* in bacterial aggregations. (A) The hatchling light organ is composed of two ciliated fields, each having a posterior (pa) and anterior (aa) ciliated appendage (inset; bar, 25 μm). GFP-labeled *V. fischeri* (arrows) formed dense aggregations outside the light organ pores when incubated in natural seawater containing a background of a variety of gram-negative and gram-positive nonsymbiotic bacteria. Bar, 10 μm. (B) An inoculum of equal concentrations of GFP-labeled *V. parahaemolyticus* (arrow) and unlabeled *V. fischeri*, viewed by LSM and DIC microscopy after a 4-h incubation, revealed dominance of *V. fischeri* in the aggregations. Bar, 10 μm. (C) Competition experiments between *V. fischeri* (V.f.) and either *V. parahaemolyticus* (V.p.) or *P. leiognathi* (P.l.), between nonmotile (nm) *V. fischeri* and *V. parahaemolyticus*, or between heat-inactivated (hi) or azide-treated (at) *V. fischeri* and *V. parahaemolyticus* ($n = 6$ aggregations for each; weighted means ± SDs are shown).

gregations (Fig. 1B and C); the nonsymbiotic strains averaged fewer than 8% of the cells. GFP labeling had no effect on this phenomenon; i.e., *V. fischeri* was dominant both as the labeled and as the unlabeled species.

In experiments designed to determine whether motility in *V. fischeri* contributed to its dominance over other species of bacteria, a *V. fischeri* strain defective in motility was the principal cell type in the aggregations after a 4-h incubation period with *V. parahaemolyticus*. Although *V. parahaemolyticus* cells were found in significantly greater numbers ($P < 0.05$; Student's *t* test), comprising up to 20% of the cells within a given aggregation (Fig. 1C), the lack of motility did not abolish the dominance of *V. fischeri*.

These data, taken together, suggest that *V. fischeri* is somehow able to outcompete other environmental bacteria for space within the aggregations, even bacterial species such as *V. parahaemolyticus* and *P. leiognathi* that can form tight aggregations on their own. Motility appears to play only a partial role in the ability of *V. fischeri* to outcompete other bacteria within the aggregations.

Growth within the aggregations. A series of experiments was performed to determine whether dominance of *V. fischeri* in the aggregation is due to more efficient growth in the mucus matrix. Incubation with nalidixic acid (25 μg/ml) inhibited *V. fischeri* cell division in culture and resulted in the formation of cells having a 17-fold increase in length (24 ± 10 μm) over untreated cells (1.4 ± 0.2 μm) (mean ± standard deviation [SD]; $n = 20$ cells). However, when animals were coincubated with 25 μg of nalidixic acid per ml and 10^6 *V. fischeri* cells per ml, cells within the aggregations were only 1.5 times longer (2.4 ± 1.2 μm) than untreated cells (1.5 ± 0.6 μm) (mean ± SD; $n = 20$ cells). While this difference represented a significantly greater cell length ($P < 0.01$; Student's *t* test), on average less than one bacterial cell division or doubling would have taken place during the 4-h incubation period in the absence of nalidixic acid. These data provide evidence that growth of the *V. fischeri* population is minimal within the aggregations during these few hours when they are associated with host-secreted mucus outside the light organ. Thus, most of the bacteria that comprise these aggregations have most likely been accumulated or harvested as individual cells from the environment.

Effect of bacterial viability on aggregation and dominance. Experiments were performed to determine whether the viability of bacteria is critical for aggregate formation and dominance of *V. fischeri* in the aggregates. Within the aggregations, the low incidence of propidium iodide staining of *V. fischeri* cells (<1%) revealed that the vast majority of the symbionts were viable under these conditions. However, *V. fischeri* cells that were heat inactivated or azide treated before inoculation stained vividly with propidium iodide, whereas viable cells in control populations did not (data not shown). Nonviable *V. fischeri* cells lost the ability to form compact aggregations (Fig. 2), although they were still capable of adherence to mucus strands. Within these loose aggregations, the distance between the center of a cell and the center of the cell of its closest neighbor was 3.0 ± 2.3 μm (mean ± SD; $n = 20$), compared to 0.6 ± 0.1 μm (mean ± SD; $n = 20$) between the cells in normal, tight aggregations. Coincubation of live *V. fischeri* with

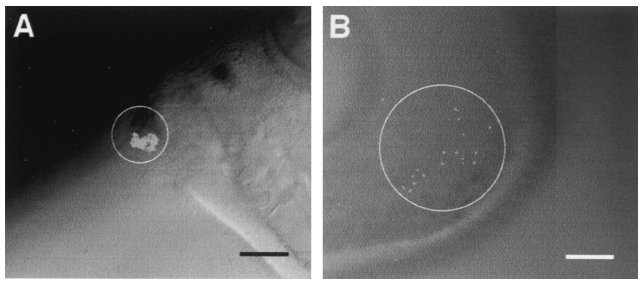

FIG. 2. Cell viability requirement for formation of tight aggregations. (A) When viable *V. fischeri* cells were added to seawater containing hatchling squid, tight dense aggregations (circle) formed above the pores on each side of the light organ, as illustrated in this LSM-DIC image of a representative animal. (B) Heat-inactivated or azide-treated *V. fischeri* cells formed significantly looser aggregations (circle) (see text for details). Bars, 20 μm.

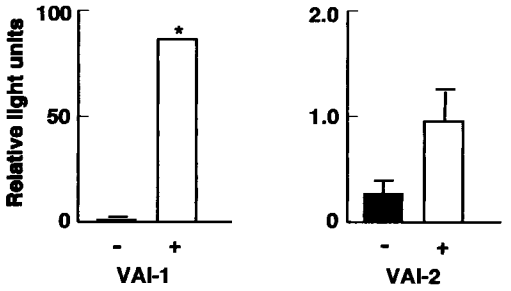

FIG. 3. Potential for induced luminescence within the aggregations. In the absence of either quorum-sensing molecule (black bars), luminescence was not detectable in the dense aggregations of *V. fischeri* NM200. After exposure of these same aggregations to either VAI-1 or VAI-2 (open bars), a significant increase in luminescence was detected ($P < 0.05$ and $P < 0.01$ [Student's t test], respectively). These results were similar to those under culture conditions where VAI-1 typically induces luminescence to a ~100-fold-greater level than VAI-2 (C. Lupp and E. Ruby, personal communication). Means ± SDs are shown ($n = 20$ for each condition). *, luminescence ranging between 8 and 250 relative light units for this condition, with all values significantly above background.

dead *V. fischeri* showed a dominance of live bacteria (>99%) within the aggregations.

Viability of *V. fischeri* cells was essential for their dominance over other bacterial species in the aggregations. When *V. parahaemolyticus* or *P. leiognathi* was presented to host animals as the sole inoculum, propidium iodide labeling revealed that the vast majority (>99%) of these nonsymbiont cells in these aggregations were viable. When *V. fischeri* was presented to animals at a 1:1 ratio with *V. parahaemolyticus* or *P. leiognathi*, both nonsymbiotic species of bacteria retained viability at >99%, although they occurred as a lower proportion of the aggregated cells over the period during which aggregates were forming, i.e., from 2 to 4 h. When live *V. parahaemolyticus* or *P. leiognathi* cells were incubated with heat-inactivated or azide-treated *V. fischeri*, the viable nonsymbiotic bacteria aggregated as they do in the absence of viable *V. fischeri* and were the dominant species in the aggregations (Fig. 1C). These data show that viability of *V. fischeri* is essential not only for the formation of tight aggregations initially but also for successfully competing with nonsymbiotic bacterial strains for space within these structures.

Potential for quorum sensing in the aggregations: luminescence. One obvious difference between *V. fischeri* and many other co-occurring environmental bacteria is the quorum-sensing system for luminescence. Thus, experiments were performed to determine whether the principal signal molecules associated with this process in *V. fischeri*, the acyl homoserine lactones VAI-1 and VAI-2, accumulate in the aggregations and may thereby be a potential signal controlling dominance of the symbiont in those sites. By 12 h postinoculation, dense aggregations of nonmotile cells of *V. fischeri*, NM200, accumulated on either side of the light organ. Although the aggregations appeared to have similar numbers of cells at a density similar to that found in the light organ crypt spaces of a fully colonized animal (approximately 10^5 to 10^6 cells), which would exhibit induced luminescence, no luminescence was detected (Fig. 3). However, when these animals were incubated with nonmotile mutants and exogenous autoinducers, large aggregations of bacteria from which light production was detectable were formed (Fig. 3); i.e., these cells were capable of responding to exogenously added autoinducers but were not accumulating them within the aggregations at levels that would promote luminescence. Thus, VAI-1 and VAI-2 do not appear to be the signal controlling dominance of the symbionts in the mucus aggregations.

DISCUSSION

Previous studies of the squid-vibrio system had revealed that the colonization of the newly hatched squid is a temporally and spatially complex process resulting in an exclusive partnership that ensues within hours of the host's hatching (11, 27). The present study provides evidence that this specificity begins before the bacterium enters the tissues of the host light organ, with the interactions of the bacterial symbiont with nonspecific bacteria. The symbiont actively establishes competitive dominance in the host-secreted mucus matrix in which harvesting of potential bacterial partners takes place.

When present in seawater that does not contain *V. fischeri* cells, the juvenile host animal will exclude all other species of bacteria from its light organ. However, *V. fischeri* is typically present in the habitat of *E. scolopes.* Under these conditions, the bacterial symbiont participates directly and actively in the establishment of specificity by its behavior within the mucus. Previous characterizations of the initial stages of the squid-vibrio association had shown that the first steps in the process involve relatively nonspecific responses of the host to environmental bacteria (20, 21). Both gram-negative and -positive bacteria, as well as their shared cell-envelope molecule peptidoglycan, induce shedding of mucus from the ciliated surface of the organ; this mucus will form the matrix where potential symbionts will gather (20). However, only gram-negative bacteria in the environment are capable of adhering to this mucus matrix (21).

The results of the study presented here demonstrate that *V. fischeri* dominance within this matrix provides the next step in the progression of ever-increasing specificity in this association. It is this step that focuses the specificity at the bacterial species level. The subsequent steps in the process, i.e., the negotiation of the duct and colonization of the crypts, require specific capabilities by the *V. fischeri* cells, such as normal flagellation and motility (4, 16), but these steps are downstream of the establishment of the exclusive partnership between *E. scolopes* and *V. fischeri*. Once inside the light organ, competitive interactions between naturally occurring strains of *V. fischeri* result in the dominance of strains with higher fitness in the light organ (9, 19, 26, 29).

How *V. fischeri* is able to outcompete other bacteria in the mucus aggregations remains to be determined and can be effectively addressed by molecular genetic studies of the symbiont (23, 28). However, our findings provide clues to the mechanism(s) underlying dominance in host mucus. In the absence of *V. fischeri,* nonsymbiotic species of bacteria form tight aggregates of viable cells; thus, the host mucus itself is not composed of a substrate(s) in which only *V. fischeri* can persist. Further, the data do not support the involvement of either superior symbiont growth or symbiont killing of nonsymbiont cells in the matrix.

In the absence of the symbiont, the formation by nonsymbiotic species of aggregations that are indistinguishable from those formed by the symbiont suggests that superior adhesion by *V. fischeri* to host mucus is unlikely, although this possibility cannot be ruled out entirely. Such an ability of one bacterial species to outcompete another for adhesion sites on mucus, or to displace a population of bacteria already adhering to mucus, is not uncommon among bacterial species (6, 10, 25). Alternatively, *V. fischeri* cells may actively promote their dominance by altering the chemistry of the mucus matrix itself, creating a biofilm that favors their persistence at the expense of that of other bacteria.

Viability was critical for the formation of tight aggregations of *V. fischeri* in the mucus matrix, suggesting that the process does not result from a passive adhesion but rather requires metabolic activity of the bacterial cells. Further, our results demonstrate that tight aggregation of *V. fischeri* cells is required for their dominance in the mucus over other bacterial species. While the nature of the required metabolic activity remains undetermined, it appears not to involve either motility or quorum-sensing-driven luminescence. Attempts to detect luminescence in the aggregates failed, although these cells could respond to exogenous autoinducer (Fig. 3). Perhaps the ciliated currents created by the host inhibit the necessary buildup of a soluble factor such as autoinducer. However, the possibility that some other autoinducer of *V. fischeri* may be involved in aggregation should not be ruled out. Our finding that cells in the aggregations are not fully induced for luminescence supports earlier reports that wild-type *V. fischeri* does not have a competitive advantage over quorum-sensing mutants during the initial stages of the symbiosis (26). Similarly, motility is not required for the formation of tight aggregations and dominance in the matrix, suggesting that while motility and chemotaxis may be essential for the subsequent stages of colonization (4, 16), they are not critical for the establishment of specificity.

Mucus substrates are among the most common matrices colonized by bacteria (22). Numerous studies of the behavior of bacteria within such environments have revealed that they often participate in the formation and maturation of a biofilm that further promotes microbial growth and persistence. These biofilms commonly have dominant bacterial species that occupy well-defined microniches, such as colonization of the lung mucosa of cystic fibrosis patients by *Pseudomonas aeruginosa* (30) or of the normal gut mucosa by *Lactobacillus* spp. (10, 25). The process of mature biofilm formation can occur over months to years under normal conditions, and biofilms are often difficult to access or manipulate experimentally. In the squid-vibrio system, the engagement of hundreds to thousands of species of environmental bacteria is discouraged in favor of interaction with the specific symbiont over a period of between 2 and 4 h. This remarkably accelerated process of selection provides an opportunity to gain insight into the mechanisms by which bacterial specificity within other mucus matrices is established.

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